Thiyl free radical production with hematoporphyrin derivative, cysteine and light: a spin-trapping study

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Hematoporphyrin derivative, used in photodynamic therapy of cancer, was found to generate the cysteinyl free radical as seen by spin-trapping. Oxygen appears to be an absolute requirement for thiyl radical production. Singlet oxygen may be the initiating species since azide inhibits oxygen uptake and radical production. In addition, the hydroxyl radical, or a radical with similar reactivity, is also observed and is proposed as the precursor for thiyl free radical production.

Hematoporphyrin derivative *Cysteine* *Thiyl free radical* *Singlet oxygen* *Hydroxyl radical* *Spin-trapping*

1. INTRODUCTION

Photodynamic therapy with hematoporphyrin derivative (HPD) is currently being developed as a treatment for malignant disease. The dihematoporphrin ether (DHE) has been found to be the most active component of HPD [1]. Hematoporphyrin is a mixture of many porphyrins with a minimal amount of the DHE found in HPD. Authors in [2] have provided evidence that the cytotoxic agent is derived from oxygen. Cytotoxicity has been attributed to singlet oxygen [3,4], an excited state of molecular oxygen, and to free radicals [5]. In vitro studies have demonstrated the production of free radicals by hematoporphyrin [6–10], in addition to its well known ability to produce singlet oxygen.

We have used spin-trapping to examine free radical formation by HPD in the presence of cysteine. Spin-trapping is a technique in which very short-lived free radicals can be accumulated by an addition reaction to a spin-trap to produce a long-lived free radical product, spin-adduct, which can be detected by ESR (reviews [11–14]).

We report here the light-induced generation of hydroxyl and cysteinyl radicals by HPD and light in the presence of cysteine and oxygen.

2. MATERIALS AND METHODS

Photofrin II, i.e., a purified preparation of HPD with a high proportion of DHE, was purchased from Oncology Research and Development, Cheektowaga, NY, USA, and was used as received. L-Cysteine hydrochloride, catalase, and superoxide dismutase were products of Sigma, St. Louis, MO, and were used without further purification. The spin-trapping agent, DMPO (5,5-dimethylpyrroline *N*-oxide), was a product of Aldrich, Milwaukee, WI. The DMPO was purified as in [15] and stored at 4°C as an aqueous solution.

The concentration of the DMPO stock solution was determined using $\epsilon_{232} = 7700\ M^{-1} \cdot cm^{-1}$ in ethanol [16]. In all experiments, an ~1:100 dilution in 50 mM phosphate buffer (pH 7.8) was made of the Photofrin II solution as supplied. This resulted in an absorbance of ~2.0 for the Photofrin II at 365 nm. The absorbance of Photofrin II has been reported to be 102 for a solution containing 1 mg/ml [17].

ESR spectra were obtained with a Varian E-109B ESR spectrometer using an aqueous sample cell and the E-238 cavity. Oxygen uptake was monitored with a Yellow Springs Instrument Model 53 Biological Oxygen Monitor. A 100 W

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quartz tungsten-halogen lamp (Oriel) operating at 3200 K was employed as a white light source. The light incident on the sample was filtered through an Oriel IR blocking filter (no. 5205) and an Oriel long pass filter (no. 5130, 50% transmission cut at 530 nm). Using a Yellow Springs Instrument Model 65A radiometer and 6551 probe, the filtered light intensity was determined to be 480 $J \cdot m^{-2} \cdot s^{-1}$ for the oxygen uptake experiments and 560 $J \cdot m^{-2} \cdot s^{-1}$ for the ESR experiments as measured 1 cm in front of the cavity grid. The cavity grid transmits 75% of the incident light.

3. RESULTS

3.1. *Oxygen uptake*

As seen in fig.1, oxygen is consumed when an air-saturated cysteine/HPD solution is irradiated with filtered white light. At points 'x' in fig.1 A–D, the light was turned off and catalase was added. As no return of oxygen was observed, no hydrogen peroxide appears to have been present. Curve E shows a decrease in the rate of oxygen consumption with the addition of 5 mM N_3^- to the cysteine/HPD solution. A similar decrease was observed with 2 and 10 mM N_3^- (not shown). No oxygen was consumed in an irradiated HPD solution in the absence of cysteine.

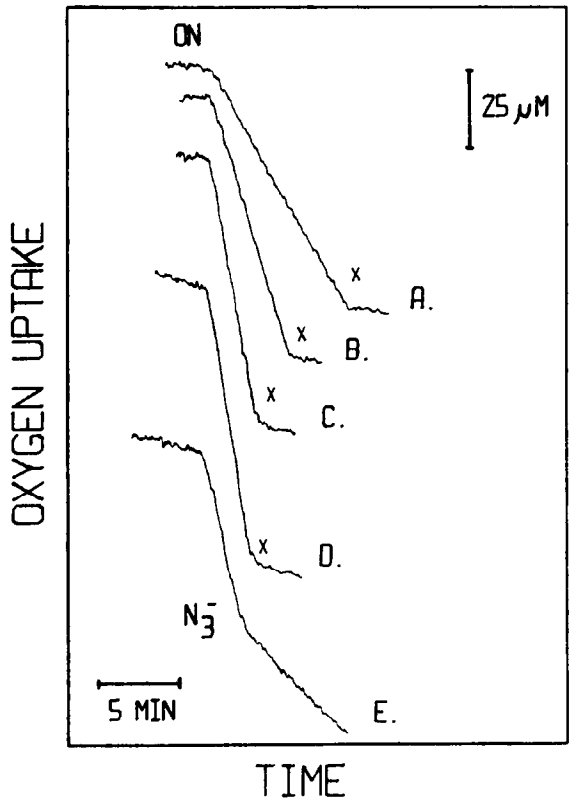

Fig.1. Oxygen uptake of air-saturated solutions containing HPD (~1:100 dilution of Photofrin II, absorbance = 2 for a 1 cm path at 365 nm) and cysteine in pH 7.8 phosphate buffer. Irradiation with filtered white light was initiated at 'ON' for each solution. For solutions A–D the light was turned off at 'x' and 1000 units/ml of catalase was added. Cysteine concentrations were: (A) 1 mM, (B) 5 mM, (C) 10 mM, (D) 25 mM, and (E) 5 mM. Azide at a final concentration of 5 mM was added at the break in curve E as irradiation continued. No rapid uptake of oxygen was observed in an irradiated HPD solution in the absence of cysteine (not shown).

3.2. *Spin-trapping*

The DMPO/Cys-S$^{\cdot}$ (Cys-S$^{\cdot}$ = cysteine free radical) and DMPO/$^{\cdot}$OH spin adducts are observed depending on reaction conditions. The ratio of DMPO/Cys-S$^{\cdot}$ to DMPO/$^{\cdot}$OH increased as the cysteine concentration was increased from 1 to 25 mM with DMPO at 50 mM (fig.2A–C, fig.3A). In fig.2D, when cysteine was 25 mM and DMPO was reduced to 10 mM, only DMPO/Cys-S$^{\cdot}$ was observed with no detectable DMPO/$^{\cdot}$OH.

The inclusion of catalase and superoxide dismutase in the air-saturated HPD/cysteine/DMPO solution resulted in no change in the nature or ratio of spin adducts observed (fig.3A,B).

When EtOH was added to the cysteine/HPD spin-trapping solution, the DMPO/$^{\cdot}$OH radical was reduced in intensity and a carbon-centered radical was present with hyperfine splitting constants consistent with those of the α-hydroxyethyl free radical (fig.3E).

Oxygen is an absolute requirement for the observation of spin-trapped radicals: when the solution is thoroughly bubbled with nitrogen before irradiation no spin-trapped radicals are detected (fig.3C). The inclusion of azide in the spin-trapping solution reduced the concentration of the trapped radicals with no evidence for DMPO/$N_3^{\cdot}$ adduct in the spectra, as shown in fig.3D.

No ESR signals were observed if either HPD or cysteine were absent in the irradiated solutions. No loss of HPD was observed as noted by monitoring the absorbance of HPD at 365 nm after irradiation

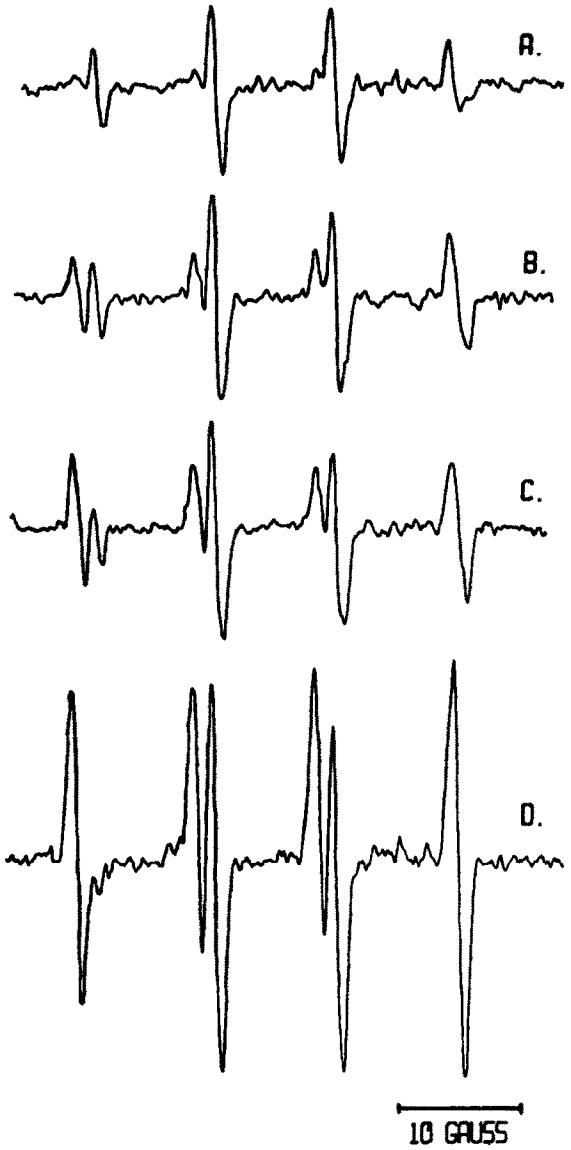

Fig.2. Spin-trapping of radicals in irradiated HPD/cysteine solutions with DMPO. HPD was present as a ~1:100 dilution of Photofrin II in air-saturated phosphate buffer, pH 7.8. Solutions consisted of: (A) 1 mM cysteine, 50 mM DMPO, (B) 10 mM cysteine, 50 mM DMPO, (C) 25 mM cysteine, 50 mM DMPO, (D) 25 mM cysteine, 10 mM DMPO. Spectrum A is that of the DMPO/ OH spin adduct ($a_N = a_H = 15.0$ G [12,24]). Spectrum D ($a_N = 15.3$ G, $a_H = 17.25$ G) has been identified as the DMPO/Cys-S$^{\cdot}$ spin adduct [25,26]. Irradiation of the sample in the cavity began approximately 5 min before the scan encountered the low field lines (left). Instrument settings were: power, 10 mW; modulation amplitude, 0.5 G; gain, 3.2×10^4; scan was 100 G/4 min; time constant, 0.5 s.

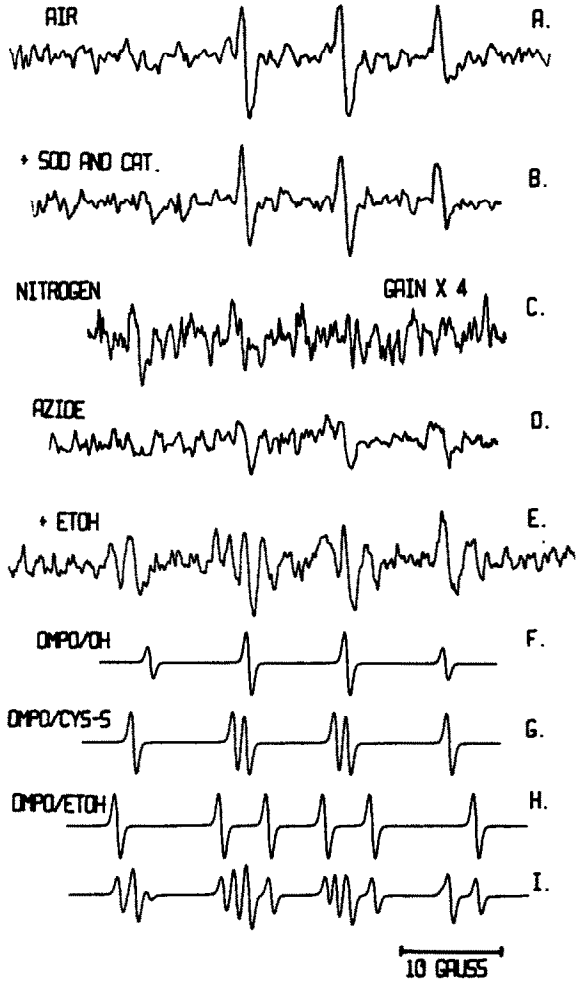

Fig.3. Spin-trapped radicals in irradiated HPD/ cysteine solutions. All experimental spectra, A–E, contained 50 mM DMPO, 5 mM cysteine with HPD present as a ~1:100 dilution of Photofrin II in phosphate buffer, pH 7.8. (A) Air-saturated solution, (B) air-saturated solution with superoxide dismutase (100 units/ml) and calatase (1000 units/ml) present, (C) nitrogen-saturated solution (note that the gain is increased by a factor of 4), (D) azide present at 5 mM, (E) EtOH present at 2 M. Instrument settings were as in fig.2. However, the scan and sample irradiation were started simultaneously. Thus, the low field lines (left) were encountered approximately 1 min after irradiation began. Curves F–I are computer-simulated examples of the species present. (F) DMPO/OH, $a_N = a_H = 15.0$ G [12,24], $g = 2.0047$ [25], (G) DMPO/Cys-S$^{\cdot}$, $a_N = 15.3$ G, $a_H = 17.25$ G, $g = 2.0047$ [25,26], (H) DMPO/EtOH, $a_H = 22.9$ G, $a_N = 15.8$ G [12], $g = 2.0053$; (I) all 3 species are present with relative intensity ratio of 1:6:4, DMPO/OH:DMPO/Cys-S$^{\cdot}$:DMPO/EtOH.

of an air-saturated solution until less than 10% of the dissolved oxygen remained.

4. DISCUSSION

A mechanism consistent with these observations is (HPD* (S_1) = excited singlet HPD and HPD* (T) = excited triplet HPD):

$$\text{HPD} + h\nu \rightarrow \text{HPD}^* (S_1) \rightarrow \text{HPD}^* (T) \quad (1)$$

$$\text{HPD}^* (T) + {}^3O_2 \rightarrow \text{HPD} + {}^1O_2 \quad (2)$$

$$\text{Cys-SH} + {}^1O_2 \rightarrow \text{Cys-}\overset{+}{\underset{H}{S}}\text{-OO}^- \rightarrow \text{Cys-SOOH} \quad (3)$$

$$\text{Cys-SOOH} \rightarrow \text{Cys-SO}^{\cdot} + {}^{\cdot}\text{OH} \quad (4)$$

$${}^{\cdot}\text{OH} + \text{Cys-SH} \rightarrow H_2O + \text{Cys-S}^{\cdot} \quad (5)$$

$$\text{DMPO} + {}^{\cdot}\text{OH} \rightarrow \text{DMPO/OH} \quad (6)$$

$$\text{DMPO} + \text{Cys-SO}^{\cdot} \rightarrow \text{DMPO/Cys-SO}^{\cdot} \quad (7)$$

$$\text{DMPO/Cys-SO}^{\cdot} + \text{Cys-SH} \rightarrow \text{DMPO/OH} + \text{R-S-S-R} \quad (8)$$

$${}^{\cdot}\text{OH} + \text{EtOH} \rightarrow H_2O + {}^{\cdot}\text{EtOH} \quad (9)$$

$${}^{\cdot}\text{EtOH} + \text{DMPO} \rightarrow \text{DMPO/EtOH} \quad (10)$$

$$\text{DMPO} + \text{Cys-S}^{\cdot} \rightarrow \text{DMPO/Cys-S}^{\cdot} \quad (11)$$

The observation that oxygen is an absolute requirement for radical production and that azide inhibits oxygen uptake and radical formation is consistent with singlet oxygen being the species which initiates the free radical chemistry observed. Since no DMPO/Cys-S$^{\cdot}$ was observed in the absence of oxygen, the reduction of HPD(T) by cysteine appears to play only a minor role in these processes. Moreover, reduced HPD would be expected to produce superoxide, as observed for reduced crystal violet [18]. No evidence for $O_2^{\cdot -}$ or H_2O_2 was found. Addition of $^1O_2$ to methionine [19] and other sulfides [20] is known. However, the reaction of $^1O_2$ with thiols has not yet been studied in detail. We propose a reaction similar to that suggested for $^1O_2$ with methionine in [19], reaction 3. The observation of the α-hydroxyethyl free radical when EtOH is present in the HPD spin-trapping solution is consistent with the presence of $^{\cdot}$OH or a radical with similar reactivity. The cysteine peroxide formed in reaction 3 should be quite suspectible to thermal cleavage as suggested in reaction 4. (The - SOOH would exhibit instabilities similar to that of a trioxide.) The DMPO/Cys-SO$^{\cdot}$ spin adduct could rapidly react with another cysteine to yield DMPO/OH and Cys-S-S-Cys. Cys-SOH has not been isolated, probably because of its rapid reaction with cysteine [21,22].

The kinetic competition experiment results seen in fig.2 demonstrate that Cys-S$^{\cdot}$ is not the primary radical formed, but rather the end paramagnetic product. The initial radical appears to be the oxygen-centered radical trapped which yields DMPO/OH.

In summary, the following results of this investigation are relevant to the mechanism of HPD photosensitization in photodynamic therapy of tumors: (a) oxygen is an absolute requirement for free radical production with cysteine, consistent with the in vitro observations of [2]; (b) a very reactive oxygen-centered radical which may be the hydroxyl radical is produced; (c) oxygen is consumed and thus cysteine is oxidized. In addition, an unknown free radical component of singlet oxygen-thiol chemistry has been uncovered, the details of which need to be investigated.

Photodynamic therapy with HPD does not result in immediate observable damage to cells as observed microscopically. Rather, observable damage is noted several hours later. Photodynamic therapy initiates the process. Part of this initiation event could be the destruction of the small molecule cellular antioxidants. Ascorbate was observed to be oxidized by HPD [23] and vitamin E is also destroyed by HPD and light (unpublished). These reactions with cysteine suggest that glutathione is also a target for oxidation by photodynamic therapy. Thus, the free radical chain reactions that are initiated by photodynamic therapy (as seen by reactions 6, 9 and 10 of this study) could quite easily be propagated in a cellular environment with its antioxidant capacity compromised. Thus, a rapid free radical chain 'oxidation' is initiated but is left unchecked and cell death occurs.

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